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Part-3 Refined fractionation and primary TIE studies

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OVOC Reports Phase-2:

Chlorinated Micropollutants in Aquatic Effluents

- Part-1 Screening studies (WP5&6)
- Part-2 Biodegradation studies (WP8)
- *Part-3 TIE studies (WP7)*
- Part-4 In-plant TIE Studies (WP9)

Chlorinated Micropollutants in Atmospheric Emissions

Chlorinated Micropollutants in Products

Contents

Summary

To reduce the complexity of the extracts that were selected for the primary TIE study, an HPLC fractionation method using gradient elution was implemented. This method was applied to the responsive fractions selected from the aquatic effluent screening study (Workpackage 5/6). For each medium or high log Kow fraction, four further refined fractions were generated with a log Kow interval that comprises one log Kow unit. These refined fractions were tested for dioxin like response using the DR-CALUX assay and the carp hepatocyte EROD activity test.

Because initially only two fractions were responsive in the DR-CALUX assay, it was decided to concentrate a selected number of refined fractions by a factor of 50. For budgetary reasons, only those refined fractions were selected that have a log Kow > 5, and whose original fractions in the screening phase exhibited a response between the limit of detection (LOD) and limit of quantification (LOQ) (27 and 89 pg TCDD-TEQ/litre effluent, respectively). The extracts that were concentrated were the 4 re-fined fractions from the high log Kow extracts of the samples MAB, MCD, MEF, MIJ, MMN and MST, as well as the medium log Kow extracts of the samples MDE, MEF and MMN.

The pattern that arises from the results of the DR-CALUX assay on these concentrated refined fractions is that for all samples, dioxin like activity was focused in the fraction with the highest possible log Kow interval for that extract. For example, activity was found in the samples MAB.W.2602.LL.high, log Kow > 8, or MEF.W.1402.LL.medium, log Kow > 6. Only sample MCD shows response in an additional fraction, namely MCD.W.1202.LL.medium, log Kow 5-6.

No strong correlation could be established between the DR-CALUX and EROD activity results, because the responses in the EROD test were too low to be significantly different from control samples. Retesting the 50 times concentrated refined fractions in the EROD assay in order to qualitatively confirm the DR-CALUX results was not carried out be-cause of limitations of sample size and budgetary constraints.

A selection of seven responsive concentrated fine fractions was also subjected to the DR-CALUX test after acidic (sulphuric acid) cleanup. After this acidic cleanup the response was quantifiable (above the limit of quantification, LOQ, of 0.8 pg TEQ/L) in 4 out of seven fine fractions with concentrations in the effluent ranging from 0.9 – 11 pg/L, and detected above the limit of detection (LOD, 0.2 pg TEQ/L) in 2 fine fractions. The acidic cleanup resulted usually in a strong reduction of the DR-CALUX response to les than 2% - 14% of the response before cleanup. In one sample (MST) this reduction was less (41% of response before cleanup).

An important additional result from this primary TIE study is the stability of the DR-CALUX response for effluents that have been kept in storage at -18 °C for the duration of approximately 1 year.

The conservation of the DR-CALUX response is clear from the comparison of the assay results from the screening phase (2001) with those obtained on the freshly prepared fractions using the effluent samples that had been kept in cold storage (2002).

This implies that the responsive compounds were stable and that the long-term repeatability of the DR-CALUX is sufficiently robust for the present study.

Identification of compounds in the concentrated refined fractions was done with GC-MSD (gas chromatography with mass spectrometric detection) using AMDIS software to deconvolute the large GC-MSD data sets into signals that could be identified using the NIST library. When presence of chlorine is applied as a criterion, a very small percentage of compounds tentatively identified by chemical analysis remains.

After manual check of the mass spectra for the presence of chlorine isotope patterns, no compounds could be identified undisputedly as containing chlorine. A large number of compounds still remain unidentified, but manual interpretation of all the related mass spectra is beyond the scope of the project.

Additional verification with GC-NCI-MS (gas chromatography with negative chemical ionization mass spectrometry), a technique more sensitive for chlorinated compounds than the GC-MSD screening method (with electron impact ionization), confirmed the predominance of non-chlorinated compounds and did not reveal significant amounts of chlorinated compounds above the limit of detection.

1. Introduction

Following sampling, pre-treatment and classification (Workpackage 5) and primary effect screening (Workpackage 6), refined fractionation and primary TIE studies are carried out as part of the OVOC Workplan Main Study (Phase 2). The results of the proceeding Workpackages 5 and 6, described in a draft report (Lamoree et al., 2003), form the basis for the more detailed investigations described in Workpackage 7. In Workpackages 5 and 6, a data set was created containing, chemical and toxicological data related to 21 samples that were roughly fractionated into three log K_{ow} classes, namely low (log K_{ow} < 4), medium (log K_{ow} 4-6) and high (log K_{ow} > 6). Among the 21 samples were industrial effluents, cooling water in/outlet, effluent from a municipal wastewater treatment plant, surface water and a blank.

In cooperation with the BOVOC supervising committee at the technical workshop held on 30 August 2001 in The Hague, a number of 10 fractions were chosen for further research regarding refined fractionation and primary TIE studies. The criteria that were applied were (see also Lamoree et al. (2002) p. 57-59):

- The presence of chlorinated compounds (EOX, AOX, GC-ECD/MSD);
- Toxic response in *in-vivo* and *in-vitro* assays;
- Bio accumulative potential, by evaluating the fractions of medium and high hydrophobicity: log K_{ow} 4-6 and log K_{ow} > 6;
- Significance of emission based on discharge volume of the effluent (by analysing annual loads rather than concentrations).

Based on these criteria, 10 fractions (see Table 1.1) were chosen to enter Workpackage 7. In addition, the medium log K_{ow} MMN sample was added as a reference for this log K_{ow} range, making a total of 11 fractions for Workpackage 7.

Table 1.1 Fractions included in primary TIE studies.

Sample	Medium log K_{ow} fraction	High log K_{ow} fraction
MCD	X	X
MDE		X
MEF	X	X
MIJ		X
MQR		X
MAB		X
MMN (MWTP)	X	X
MST (cooling water outlet)		X

Workpackage 7 comprises refined fractionation of the 11 chosen fractions into sub fractions covering a narrower log K_{ow} interval, followed by chemical analysis, confirmation of *in vitro* activity and chemometric analysis (multivariate statistical analysis to relate effects to presence and concentrations of identified compounds) (Van Hattum et al., 2000). This is schematically represented in Figure 1.1.

As it became clear that no chlorinated compounds were detected with GC-MSD screening technique in the final responsive fractions, the chemo-metric data analysis was not

executed. A selection of responsive samples was analysed with GC-NCI-MS (with negative chemical ionisation), which was carried out by the Netherlands Institute for Fisheries Research (RIVO). This technique is much more selective and sensitive for chlorinated compounds than the electron impact based GC-MS method. Regarding the in vitro assays, extra DR-CALUX testing of responsive fractions was done before and after acidic cleanup of the extracts.

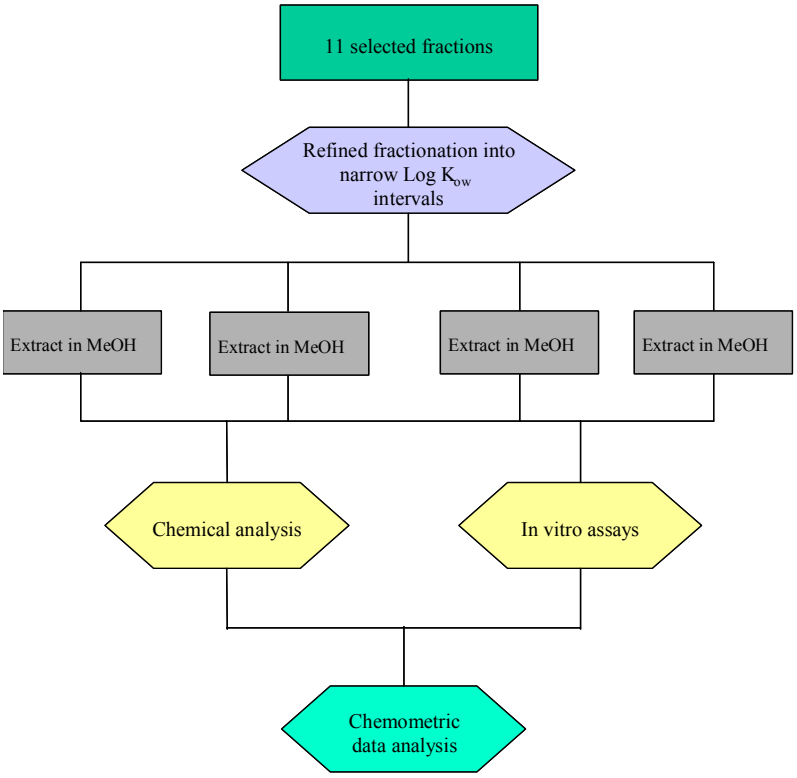

Figure 1.1 Flow scheme of refined fractionation and primary TIE studies.

During validation of the isocratic fractionation of samples with methanol into 3 log K_{ow} classes, described in the Draft Report WP5&6, it was discovered that recoveries of test compounds were low with the HPLC system that was used for the preparation of the extracts. Much better recoveries were achieved with manual instead of automated injection of the samples to be fractionated. Therefore, the 11 extracts that were selected for primary TIE studies were freshly prepared with the new method, using effluent that had been stored at -18 ˚C since the time of sampling. In addition to that, a blank was included consisting of HPLC water.

This report is intended to become an part of the Technical Annex of the final report on the Chlorine Chain Follow-up Studies, and should be read as a logical follow-up of the draft report on Workpackages 5 and 6, in which the preceding research activities are described in detail. Eventually, when the research programme has come to an end, a final report will be assembled that will consist of the combined reports on the various workpackages.

2. Refined fractionation (task 7.1)

2.1 Introduction

Refined fractionation of the extracts that were investigated in Workpackages 5 and 6 is required in order to obtain more detailed information on for instance the true log K_{ow} range of extracts that are responsive in the chosen bioassays. Another goal is to decrease the complexity of the composition of the extract and therefore to simplify the relation between toxic effects and the results of chemical analysis.

Before extracts were subjected to refined fractionation by means of reversed phase HPLC, the fractionation method was validated with a set of test compounds, in analogy to the fractionation method that was used to produce extracts corresponding 3 hydrophobicity classes (low, medium and high) in Workpackage 5.

Refined fractionation of a single extract that was chosen for Workpackage 7 generates a number of 4 new fractions with a narrow log K_{ow} range. For these refined fractions the DR-CALUX and carp hepatocyte EROD induction assays (task 7.2) and chemical analysis (task 7.3) were carried out.

2.2 Refined fractionation

2.2.1 Reversed phase HPLC method

For refined fractionation according to hydrophobicity, a reversed phase HPLC method was implemented. The separation column was a 250 x 4.6 mm Vydac TP254 C18 column, particle size 5 µm, in combination with a guard column. A linear gradient was used, starting at 50/50 methanol/H_2O (v/v) to 100 % methanol after 50 min. This method was described earlier by Verbruggen et al. (1999) for separation of complex mixtures of organic micropollutants according to their hydrophobicity. The injection volume was 100 µl, the column temperature was 22 ˚C and the flow rate was 1 ml/min. For fraction collection, a Foxy 200 X-Y fraction collector was used.

The starting material for refined fractionation consisted of methanolic extracts that resulted from isocratic fractionation using a C18 reversed phase column and methanol, as described in Chapter 4 of the Draft report WP5&6. With such a system, only a very rough fractionation with regard to log K_{ow} is achieved (Klamer et al., 1995). The log K_{ow} intervals for those fractions were defined as medium log K_{ow} (roughly between log K_{ow} 4 and 6) and high log K_{ow} (roughly above log K_{ow} 6). In order not to lose any compounds from these fractions that might elute outside this log K_{ow} window in a refined fractionation set up, the method was designed to cover for compounds with lower or higher log K_{ow} than the defined window. In Figure 2.1, a schematic representation of the refined fractionation into narrow log K_{ow} intervals is given.

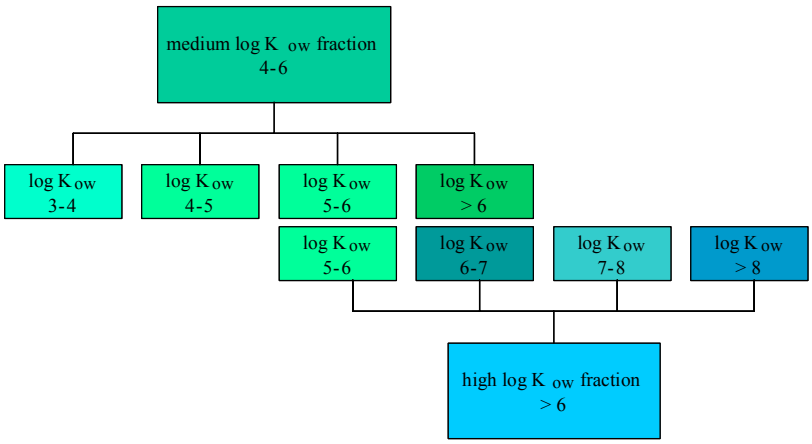

Figure 2.1 Schematic representation of the refined fractionation scheme, starting from medium and high log K_{ow} fractions.

To establish the switching time points of the fraction collector, a linear relationship between log K_{ow} and retention time was determined for a mixture of compounds, consisting of a series of polyaromatic hydrocarbons (from NIST standard 1647d), polychlorinated biphenyls and organochlorine pesticides. The log K_{ow} of these compounds ranges from 1.14 for chloridazon to 8.27 for PCB 180. The linear relationship and the derived switching times for the fraction collector to obtain the proper log K_{ow} intervals is described in Appendix I.

As a logical consequence of the linear relationship, the volume of all the refined fractions is approximately the same, because they all cover a single log K_{ow} unit, except the refined fractions with log K_{ow} > 6 and log K_{ow} > 8. For these two fractions, the HPLC effluent is collected up to 92 minutes, resulting in fractions with volumes of 57 and 44 ml for log K_{ow} > 6 and log K_{ow} > 8, respectively.

The volume of the fractions covering a log K_{ow} interval of one unit is 6 ml. These fractions have been liquid/liquid extracted with 2 x 10 ml pentane, with the pentane phase subsequently being evaporated and finally taken up in methanol.

Because of the relatively large volumes of the fractions with log K_{ow} > 6 and log K_{ow} > 8, fractions were collected in several vials. As a result of the gradient used for chromatographic elution, some vials contained a mixture of methanol and water, while others contained only methanol. For the mixed solvent fractions, liquid/liquid extraction with pentane and transfer to methanol was done in the same way as for the smaller fractions covering one log K_{ow} unit. Finally, all methanolic fractions were combined and evaporated by rotary evaporation. As in Workpackage 5, all extracts were equivalent to 1 litre effluent.

2.2.2 Validation results

In order to assess the performance of the refined fractionation by reversed phase HPLC, a mixture of PCBs and chlorinated pesticides was injected and fractionated in log K_{ow} intervals of one unit.

For this experiment, all compounds were present in a concentration of ± 65 ng/ml. For fractionation of a 500 µl sample, four times 125 µl is manually injected (see p. 17 of Draft report WP5&6) and after fractionation collected in separate vials. An extra 125 µl of solvent, used to wash the vial, is injected to enhance quantitative transfer of the extract onto the fractionation column. The collected fractions were liquid/liquid extracted and analysed by GC-ECD for quantification and recovery. In addition, a check was made whether a compound eluted in the correct log K_{ow} interval. The validation experiments were carried out in duplicate. In Table 2.1 the results with regard to recoveries and fractionation are presented.

Because of the relatively large differences in recoveries between the series for some compounds, results have not been averaged but given for each series. Recoveries for QCB, HCB and heptachlor (highlighted in Table 2.1) are exceptionally bad. These compounds are very volatile, having Henry's Law constants ranging from 29 to 172 $Pa.m^3/mol$ (see Draft Report WP5&6), and therefore they will be lost during evaporation steps in the procedure. The compounds α- and γ-HCH also show very low recoveries, while β-HCH appears in a fraction with too low log K_{ow} (also highlighted). The decreased reliability of the refined fractionation in the low log K_{ow} range is not expected to cause problems, because these compounds are known not to bio accumulate.

Table 2.1 Validation results of the refined fractionation.

compound	total recovery		log Kow	compound found in fraction:	
	serie 1	serie 2		serie 1	serie 2
g-HCH	n.d.	5, 1	3.7	n.d.	3 to 4
b-HCH	54	57	3.8	< 3	< 3
a-HCH	7.7	3,5	3.8	3 to 4	3 to 4
Telodrin	41	30	4.5	5 to 6	5 to 6
cis-HEPO	71	71	5.0	4 to 5 and 5 to 6	4 to 5 and 5 to 6
trans-HEPO	73	72	5.0	5 to 6	5 to 6
QCB	7.5	2.9	5.2	3 to 4 and 6 to 7	6 to 7
Endrin	85	101	5.2	5 to 6	5 to 6
Dieldrin	86	98	5.4	5 to 6	5 to 6
PCB 28	52	66	5.6	6 to 7	6 to 7 and 7 to 8
HCB	3.7	17	5.7	7 to 8	7 to 8
PCB 52	45	30	6.1	5 to 6	5 to 6 and 6 to 7
Heptachlor	10	n.d.	6.1	5 to 6	n.d.
Aldrin	43	55	6.5	6 to 7	6 to 7
Isodrin	54	65	6.8	6 to 7	6 to 7
PCB 101	62	77	6.8	6 to 7	6 to 7
PCB 118	62	99	7.1	7 to 8	7 to 8 and > 8
PCB 138	77	103	7.4	6 to 7 and 7 to 8	6 to 7 and 7 to 8
Octachloorstyrene	17	64	7.5	7 to 8	7 to 8
PCB 153	70	96	7.8	6 to 7 and 7 to 8	7 to 8 and > 8
PCB 180	76	105	8.3	7 to 8	7 to 8 and > 8
PCB 103	69	84	?	6 to 7 and 7 to 8	6 to 7, 7 to 8, > 8

Most compounds from the test mixture were collected in the fraction with the interval corresponding to their log K_{ow} value, but elution in the next fraction also occurred, for instance for PCB 28. Elution of compounds in two or even three (for PCB 103) adjacent fractions was also observed for a number of compounds, such as some of the PCBs.

The compounds p,p-DDT, o,p-DDT, p,p-DDD, o,p-DDD and o,p-DDE were initially included in the test mixture, but not mentioned in the table because of difficulties with quantification due to structural transformations during analysis.

3. In-vitro screening of activity in refined fractions (task 7.2)

3.1 Introduction

With the refined fractions that were prepared from the eleven freshly made extracts, DR-CALUX and carp hepatocyte EROD induction assays were carried out. In addition to the 44 fractions (11 extracts x 4 fractions) for the primary TIE studies, 8 fractions originating from HPLC water were included. As a starting point for the refined fractionation of the blank extracts, the two extracts with medium and high log K_{ow} were chosen, resulting in 2 x 4 = 8 refined fractions.

The DR-CALUX assay was carried out for the 52 refined fractions as well as the 11 freshly prepared fractions from which the refined fractions were prepared. This was done to enable comparison to the previously obtained results, and to assess whether the improved isocratic fractionation method with manual injection led to higher responses in the DR-CALUX assay. The carp hepatocyte EROD induction assay was done only for the 52 refined fractions.

After evaluation of the initial DR-CALUX results, a decision was made to concentrate 30 selected refined fractions by a factor of 50, in order to obtain responses well above the LOQ of the DR-CALUX assay, leading to more reliable and better quantifiable results. To enable better interpretation of the meaning of the observed responses in terms of the contribution of dioxins, dioxin-likes and other acid-stable compounds, 7 extracts were subjected to an acidic cleanup step using sulphuric acid treated silica columns, as described in the reports on WP9 (Senhorst et al., 2004) and on the validation of the acidic sample clean-up procedure for the DR-CALUX assay (Lamoree et al., 2004).

3.2 DR-CALUX assays

3.2.1 Introduction

All DR-CALUX assays have been carried out identical to the method described in the Draft Report WP5&6 (Lamoree et al., 2003). In Table 3.1, the results of the 11 freshly extracted fractions that were later subjected to refined fractionation are shown. For good comparison, the responses found in the screening phase are included as well.

The optimised fractionation method using manual injection has not changed the DR-CALUX responses for the 11 selected extracts for primary TIE dramatically. The HPLC blank fractions with medium and high log K_{ow} do not show any DR-CALUX response.

Furthermore, Table 3.1 shows that storage of the effluents at -18 ˚C for approximately 1 year has not had major influence on the DR-CALUX responses. Obviously, the responsive compounds were stable during storage and handling (freezing, defrosting). The conservation of response further implies that the repeatability of the DR-CALUX assay is sufficient to use as a tool for assessment of toxicity in a TIE study over a prolonged period of time.

Table 3.1 DR-CALUX results of 11 selected fractions in screening and primary TIE.

sample	2,3,7,8 TCDD-TEQ pg TCDD-TEQ/liter effluent	
	screening phase	primary TIE
MAB.W.2602.LL.high	50	45
MCD.W.1202.LL.medium	296	267
MCD.W.1202.LL.high	165	132
MDE.W.2102.LL.high	<LOD	27
MEF.W.1402.LL.medium	30	28
MEF.W.1402.LL.high	47	44
MIJ.W.2102.LL.high	27	27
MMN.W.0502.LL.medium	29	31
MMN.W.0502.LL.high	54	42
MQR.W.2901.LL.high	<LOD	27
MST.W.1202.LL.high	<LOD	28
HPLC water,LL.medium	not determined	< LOD
HPLC water, LL.high	not determined	< LOD

LOD = 27 pg TCDD-TEQ/liter effluent

Table 3.2 DR-CALUX response in pg TCDD-TEQ/litre effluent for the two selected fractions that tested positive after refined fractionation.

sample	DR-CALUX response pg TCDD-TEQ/liter effluent	refined fraction	DR-CALUX response pg TCDD-TEQ/liter effluent
MCD.W.1202.LL.medium	267	log Kow 3-4	< LOD
		log Kow 4-5	< LOD
		log Kow 5-6	< LOD
		log Kow > 6	197
MIJ.W.2102.LL.high	27	log Kow 5-6	< LOD
		log Kow 6-7	< LOD
		log Kow 7-8	< LOD
		log Kow > 8	27

LOD = 27 pg TCDD-TEQ/liter effluent

3.2.2 DR-CALUX of refined fractions

Of the refined fractions that were tested with the DR-CALUX assay, only two fractions gave a detectable response, of which only one was reliably quantifiable. In Table 3.2, the DR-CALUX results are given for these samples.

The limit of detection (LOD) and limit of quantification (LOQ) were determined to be 27 and 89 pg TCDD-TEQ/litre, respectively, the same values as in the screening phase. The responses of the 8 refined fractions originating from the two HPLC water blanks with medium and high log K_{ow} were all <LOD.

3.2.3 DR-CALUX of concentrated extracts

To improve the usefulness of the DR-CALUX assay for the assessment of toxicity in the refined fractions, it was decided to carry out an additional concentration step for a limited number of refined fractions. The implementation of such a concentration step is done in order to obtain a DR-CALUX response that is higher than the LOQ in the previous assays. A more reliable test result is thus generated.

A choice was made for those extracts that had exhibited a DR-CALUX response between the LOD and LOQ in the previous tests, and for budgetary reasons limited to the higher log K_{ow} fractions with log K_{ow}>5. This included the samples MAB, MCD, MEF, MIJ, MMN and MST.

DR-CALUX results of concentrated extracts without acidic cleanup

The extracts were concentrated by a factor of 50 by evaporation of methanol under a gentle stream of nitrogen. During and after the concentration step, no precipitate formation was observed. In Table 3.3 these DR-CALUX responses are shown, together with the calculated responses per litre effluent.

For all concentrated extracts, the refined fractions with log K_{ow}>8 gave a high response in the DR-CALUX assay. For the samples MCD, MEF and MMN the log K_{ow}>6 fraction originating from the medium log K_{ow} rough fractionation also showed considerable DR-CALUX response. The refined fraction coming from sample MCD.W.1202.LL.medium with log K_{ow}5-6 is the only sample with a log K_{ow}<6 showing some activity.

For all samples except MCD, the resulting DR-CALUX responses in the refined and concentrated fractions correspond well to the responses in the newly made fractions for the primary TIE study. It is unknown what phenomenon created the decrease in response of a factor of 5 in the MCD sample, but possibly the concentration step resulted in a loss of volatile components that were responsible for part of the response in the previous test.

The observation of almost all DR-CALUX activity in the highest log K_{ow} fractions is an important result that indicates that the approach followed in the screening phase of the OVOC study has not led to a breakdown of bioassay response in several fractions. Fractionation in low, medium and high log K_{ow} intervals enabled us to investigate dioxin-like effects for compounds ranging from hydrophilic to hydrophic, but nevertheless DR-CALUX activity was unambiguously found in the hydrophobic region. This implies that the rough fractionation has not resulted in a simplification of the composition of the responsive fractions, which is the primary goal of fractionation in a TIE study.

DR-CALUX results of concentrated extracts after acidic cleanup

Following an analogous approach as for the in-plant TIE studies described in the report on WP9 and the biodegradation studies (WP-8), an acidic cleanup step using sulphuric acid/silica columns was implemented in order to obtain insight in the nature of the

compounds still causing a response in the DR-CALUX assay (Murk et al., 1998; Houtman et al., 2004). Historically, this cleanup step is part of the standard procedure for the screening on polychlorinated dioxins (PCDDs) and furans (PCDFs) with HRGC/HRMS.

The DR-CALUX in combination with the acidic-cleanup has been tested and validated as a screening tool for the presence of dioxins and dioxin-likes for the regulatory monitoring of food and animal feeding materials (Van Overmeiere et al., 2001; Schoeters et al., 2004), and for the screening of harbour dredgings (Stronkhorst et al., 2002). For wastewater the method has not yet been validated; the application of the DR-CALUX method on effluents as this study is new. The implications of this cleanup procedure for the interpretation of the results are discussed in detail elsewhere (Lamoree et al., 2004).

A total of 7 extracts was selected for further DR-CALUX testing after acidic cleanup, the main criterion for selection being a positive response in the assay without the acidic treatment. Only the MMN samples and the MAB sample were not included. The results are represented in Table 3.3.

Of the seven extracts, three responses fell below the LOQ, which was 40 pg TCDD/liter in the 50x concentrated effluent. One of those - the MIJ sample, was below the LOD of 12 pg TCDD/liter effluent.

For MCD and MEF samples in the category "medium, log K_{ow}>6", quantifiable values of, respectively, 43 and 170 pg TCDD/liter 50x concentrated effluent were found. Translated to values for non-concentrated sample, this results in 0.86 pg TCDD/liter effluent for MCD and 3.4 pg TCDD/liter effluent for the corresponding MEF sample.

In the higher log K_{ow} region, also two positive responses were found in the DR-CALUX assay after acidic cleanup. For MEF and MST samples "high, log K_{ow}>8", the values for 50x concentrated effluents were 190 and 550 pg TCDD/liter, respectively. For the non-concentrated effluents, these values correspond to 3.8 and 11 pg TCCD/liter effluent.

Comparing the results after acidic cleanup with the response without this cleanup step, resulted for responsive fine fractions of MCD and MIJ in less than 2% of the original response which is resistant to acidic cleanup. For MEF (11-14%) and MST (41%) higher fractions of the original response appeared to be stable during acidic cleanup.

3.3 Carp hepatocyte (CARP-HEP) experiments

3.3.1 Introduction

Carp hepatocyte experiments were done as part of the in vitro screening (task 7.2) of 52 fine-fractionated extracts of several effluent samples from WP6 (MAB, MCD, MDE, MEF MIJ, MMN, MQR and MST), as well as two HPLC blanks.

Table 3.3 DR-CALUX response for the concentrated refined fractions without and after acidic cleanup.

Total extract		**Coarse fractions**		**Refined fractions**		
Sample	DR-CALUX response pg TCDD-TEQ/liter effluent [1]	*Fraction*	DR-CALUX response pg TCDD-TEQ/liter effluent [2]	*Fraction*	**No acidic clean-up** DR-CALUX response pg TCDD-TEQ/liter effluent [3]	**After acidic cleanup** DR-CALUX response pg TCDD-TEQ/liter effluent [3]
MAB	309	high	45	log Kow 5-6	0.6*	
				log Kow 6-7	< 0.5	
				log Kow 7-8	0.5*	
				log Kow > 8	27	n.d.
MCD	1066	medium	267	log Kow 5-6	4	0.4*
				log Kow > 6	46	0.86
		high	132	log Kow 5-6	< 0.5	
				log Kow 6-7	< 0.5	
				log Kow 7-8	0.9*	
				log Kow > 8	29	0.5*
MEF	99	medium	28	log Kow 5-6	< 0.5	
				log Kow > 6	25	3.4
		high	44	log Kow 5-6	0.9*	
				log Kow 6-7	0.8*	
				log Kow 7-8	0.8*	
				log Kow > 8	35	3.8
MIJ	122	high	27	log Kow 5-6	< 0.5	
				log Kow 6-7	1.6*	
				log Kow 7-8	1.6*	
				log Kow > 8	32	< 0.2
MMN	157	medium	31	log Kow 5-6	1.1*	
				log Kow > 6	23	n.d.
		high	42	log Kow 5-6	< 0.5	
				log Kow 6-7	< 0.5	
				log Kow 7-8	1.1*	
				log Kow > 8	36	n.d.
MST	104	high	28	log Kow 5-6	0.6*	
				log Kow 6-7	0.8*	
				log Kow 7-8	0.9*	
				log Kow > 8	27	11

1. DR-CALUX response measured in total extracts in the screening study (Lamoree et al., 2003);
2. DR-CALUX response measured in fractions of newly prepared extract for primary TIE (compare to Table 2.1);
3. DR-CALUX response per liter effluent; * concentrations between limit of detection (LOD) and limit of quantification (LOQ); LOD and LOQ valudes in 50 times concentrated extracts: LOD = 0.5 pgTCDD-TEQ/liter; LOQ = 1.9 pg TCDD-TEQ/liter for samples without acidic cleanup, LOD = 0.2 pgTCDD-TEQ/liter; LOQ = 0.8 pg TCDD-TEQ/liter for samples after acidic cleanup; n.d.: not determined.

The following measurements were made in the CARP-HEP assay:

- The induction in male carp hepatocytes in primary culture of cytochrome P4501A (CYP1A) enzymes, and thus the potential of the effluent extracts to cause dioxin-like toxicities. The induction of CYP1A enzymes is a consequence of an agonistic effect on the aryl hydrocarbon (Ah) receptor. Ethoxyresorufin-*O*-deethylase (EROD) activity is used as a measure of the catalytic activity of CYP1A.
- The potential cytotoxicity of the effluent extracts in the carp hepatocytes is determined using two methods. Firstly, using the MTT test which measures the mitochondrial succinate dehydrogenase activity (an enzyme in the tricarboxylic acid cycle, important for the generation of the cells energy. Succinate dehydrogenase reduces the substrate MTT to formazan which is measured spectrophotometrically. Secondly, cytotoxicity of the water extracts is determined by measuring lactate dehydrogenase (LDH) leakage from the cells. LDH leakage increases when the cell membrane is damaged by the treatment.

3.3.2 Methods

Carp hepatocyte isolation and culture as well as determination of EROD activity, protein content, and determinations of cytotoxicity (LDH, MTT) were carried out as described previously for earlier workpackages (Lamoree et al., 2003). LDH leakage was determined by the method of Bergmeyer and co-workers (Bergmeyer, Bent et al. 1965). Mitochondrial dehydrogenase activity was determined using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) as the substrate (Denizot and Lang, 1986).

3.3.3 Results

For all refined fractions, no significant increase in EROD activity was observed. Responses were too low for reliable quantitation of dioxin-like activity. Additional experiments on the 30 extracts that were 50 times concentrated, in analogy to the DR-CALUX assay, were not carried out due to budgetary constraints and limitations of sample size. As a result, qualitative confirmation of the DR-CALUX results by the EROD response (as in the screening phase) was not possible.

Regarding cytoxicity of the 52 fractionated effluent samples, none of the extracts were found to be cytotoxic as determined by the MTT reduction test or by LDH leakage.

4. Chemical analysis of refined fractions (task 7.3)

4.1 Introduction

The ten concentrated extracts that showed a positive response in the DR-CALUX assay were taken as a starting point for the chemical analyses by GC-MSD. Because of the complexity of the mass spectral data sets that are generated, an approach analogous to the one used for the in-plant TIE studies of work package 9 was adopted. In short, this consisted of a number of steps, starting with the interpretation of GC-MSD data by Automated Mass spectral Deconvolution & Identification System (AMDIS) software (Mallard et al., 1997). Using this software, matching of deconvoluted (extracted and background corrected) mass spectra with the NIST mass spectral database was done automatically, while from the list of possible constituents the chlorine containing compounds could be selected. As a last step, a preliminary interpretation of the generated results was done.

It should be emphasized that with this approach only those compounds that are included in the NIST database will be identified. However, as this database contains over a hundred thousand mass spectra, this is the most effective way of dealing with the enormous data sets. As a result, at this stage the focus is placed on tracing known, chlorine containing compounds that exhibit dioxin-like response in DR-CALUX and/or carp hepatocyte EROD test that was hitherto unknown.

Additional chemical analysis was done by GC-MS with negative chemical ionization (NCI). For the identification of the mass spectra from the GC-MS NCI chromatograms the NCI libary of RIVO was used. In addition, the AMDIS programme and the NIST98 library were used for the identification of chlorinated compounds. The NIST98 library contains EI spectra only whereas the NCI-chromatograms contain NCI spectra. However, for chlorinated, and also brominated compounds, a large overlap in mass spectra between EI and NCI modes exists. This is mainly due to the ionisation principle of chlorinated and brominated compounds (electron capture principle). For other than chlorinated and brominated compounds different ionisation mechanisms take place, and therefore, the mass spectra are different between EI and NCI mode. Due to the low ionisation energy of NCI, the mass spectra of chlorinated compounds often contains the molecular ion. In addition, sometimes the isotopes of chlorine (m/z 35 and 37) are found. The reported compounds matched by AMDIS/NIST were exported to Excel. Within the list of probable identifications a manual selection was made of chlorinated compounds. As a last step, a manual comparison of extracted spectra and library spectra was applied for confirmation.

Important to note is that large differences exist in the responses between chlorinated compounds using NCI; differences of a factor of 100 can be found.

4.2 GC-MSD analysis of 10 concentrated extracts

The GC-MSD method that was used to analyse the 10 concentrated extracts that showed response in the DR-CALUX assay was identical to the one used during the screening phase and can be found in the report on the work package 5 and 6 (Lamoree et al., 2003).

4.3 GC-MS NCI analysis

A selected number of fractionated extracts were analysed using GC-MS with negative chemical ionisation (NCI) at RIVO. The selected samples were the high log K_{ow} fractions with log K_{ow} >8 of samples MMN, MCD, MIJ, MST and MEF. Additionally, for samples MCD and MEF also the medium log K_{ow} fractions, fractionated to log K_{ow} > 8 were selected. Fractions were delivered by IVM in methanol. At RIVO the samples were evaporated almost to dryness, using nitrogen, and 200 µl of an internal standard (pentachlorobiphenyl, CB112) in ethyl acetate was added. The MS was used in full scan mode from m/z 34 to m/z 800, and methane was used as chemical ionisation gas. Source and transfer line temperature were 200°C and 290°C, respectively. The injection volume was 1 µl using pulsed splitless injection at 275°C. The GC oven initial temperature programme was 90°C for 3 min., followed by an increase of 30°C/min to 210°C for 20 min, then the temperature was increased with 5°C/min to 290°C for 27 min. A CP-Sil 8 column (50 m x 0.21 mm ID x 0.25 mm film thickness) or vDB-5 column (15 m x 0.20 mm ID x 0.20 mm film thickness were used. Helium was used as a carrier gas at a flow of 1.5 ml/min.

4.4 Results of the interpretation of GC-MSD data

In order to simplify the obtained GC-MS results into interpretable data sets, the software programme AMDIS was used to enable chromatographic peak recognition from complicated, noisy GC-MSD spectra. After deconvolution of the set of mass spectra resulting from the gas chromatographic analysis of a concentrated refined fraction, the software can be used to match the resulting spectra against the NIST library. With the implementation of specific software settings (see Table 4.1 and Senhorst et al., 2003), the resulting information can be classified with regard to a match factor that indicates the probability that a compound is identified correctly. As a final step, the presence of chlorine in the identified compounds can be verified. The results of this approach are shown in Table 4.1.

In Table 4.1 the filtering process from chromatographic peak via a tentative identification in the NIST library to compounds possibly containing chlorine is shown. From the identified compounds, the percentage of components that could possibly contain chlorine is very small. After manual check of the corresponding mass spectra for distinctive chlorine isotope patterns, no undisputed assignment of compounds that contain chlorine was possible.

Table 4.1 Results of the tentative interpretation of GC-MSD data using AMDIS.

sample	refined fraction	no. of components deconvoluted by AMDIS[1]	no of components with match in NIST library[2]	no of components recognized by NIST possibly containing chlorine[3]	no of components probably containing chlorine assigned by manual check
MAB.W.2602.LL.high	log Kow > 8	495	269	4	none
MCD.W.1202.LL.medium	log Kow 5-6	646	247	4	none
	log Kow > 6	844	315	11	none
MCD.W.1202.LL.high	log Kow > 8	643	311	4	none
MEF.W.1402.LL.medium	log Kow > 6	872	459	9	none
MEF.W.1402.LL.high	log Kow > 8	594	316	5	none
MIJ.W.2102.LL.high	log Kow > 8	1197	500	10	none
MMN.W.0502.LL.medium	log Kow > 6	1229	650	9	none
MMN.W.0502.LL.high	log Kow > 8	858	475	5	none
MST.W.1202.LL.high	log Kow > 8	680	374	7	none

1. Deconvolution settings: component width: 12; adjacent peak subtraction=2; resolution=high; sensitivity=medium; shape requirements=medium
2. Match factor > 60
3. Match factor > 70 or at least 3 out of first 5 hits are chlorine compounds

The mass spectra of the compounds that have not been identified using the NIST library have not been manually checked for presence of chlorine, as this would be too time consuming at this moment.

In Figure 4.1 and 4.2 the GC-MSD chromatograms of the samples MMN.W.0502.LL.medium, log K_{ow}>6 and MCD.W.1202.LL.high, log K_{ow}>8, respectively, are shown as typical examples of concentrated effluent extracts.

4.5 Results of GC-MS NCI analyses

The NCI-chromatograms of the fractionated extracts were dominated by non-chlorinated compounds, and this confirms the results observed with the GC-MSD. After automatic processing of the data a small number of chlorinated compounds (<5) was tentatively identified with the NIST library. After manual check of the mass spectra in most samples and comparison with procedural blanks for the GC-NCI-MS analysis no chlorinated compounds could be identified in the fractionated extracts of the effluents.

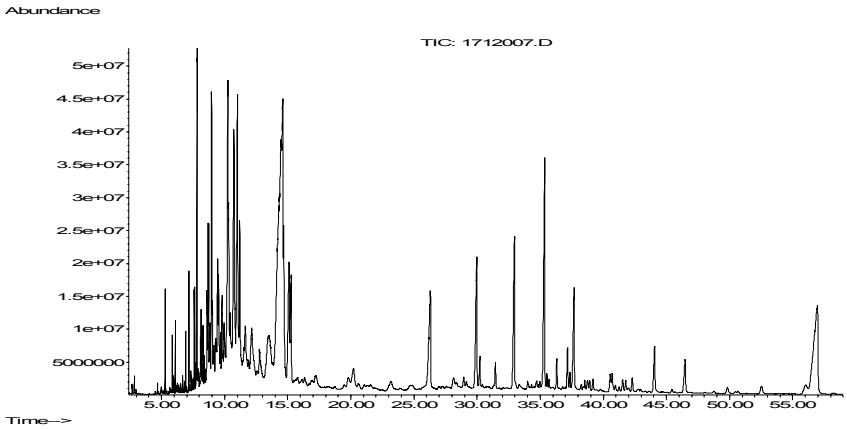

Fig. 4.1 GC-MSD chromatogram of sample MMN.W.0502.LL.medium, log K_{ow}>6, 50x concentrated.

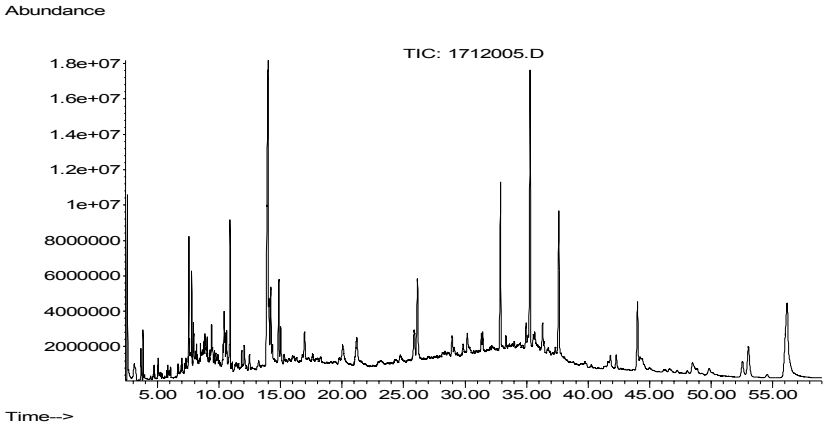

Fig. 4.2 GC-MSD chromatogram of sample MCD.W.0502.LL.high, log K_{ow}>8, 50x concentrated.

5. Conclusions

For the proposed selection of responsive fractions from the effluent screening study (Workpackages 5/6) a further fine fractionation was applied. For each medium or high log K_{ow} fraction, four further refined fractions were generated with a log K_{ow} interval that comprises one log K_{ow} unit. These refined fractions were tested for dioxin like response using the DR-CALUX and the carp-hep EROD assays.

Initially, significant dioxin-like activity as determined by the DR-CALUX assay was found in only one refined fraction, namely MCD.W.1202.LL.medium, log $K_{ow} > 6$. In one other sample, MIJ.W.2102.LL.high, log $K_{ow} > 8$, a response was found at the LOD of 27 pg TCDD-TEQ/litre effluent. No responses above the LOD were observed for the blanks. With the carp hepatocyte EROD assay, no significant responses were measured for any of the refined fractions.

The similarity of the DR-CALUX results of the newly prepared fractions that were used as starting material for the refined fractionation with the results obtained in the screening phase shows that the responsive compounds were stable during storage and that the assay is robust and reliable over a prolonged period of time, making it a valuable tool for assessment of dioxin like response in this TIE study.

In order to obtain more reliable and quantifiable results in the DR-CALUX assay, a selection of 30 refined fractions was concentrated 50 times and subjected once more to the DR-CALUX test. The results show that dioxin like activity is focused in the refined fractions with the highest log K_{ow}, either log $K_{ow} > 6$ for the medium log K_{ow} original fraction or log Kow > 8 for the high log K_{ow} original fraction. Only for sample MCD activity was also seen in another fraction, corresponding to the log K_{ow} 5-6 interval from the medium log K_{ow} original fraction.

A selection of seven responsive concentrated fine fractions was also subjected to the DR-CALUX test after acidic (sulphuric acid) cleanup. After this acidic cleanup the response was quantifiable (above the limit of quantification, LOQ, of 0.8 pg TEQ/L) in 4 out of seven fine fractions (MCD medium log Kow>6; MEF medium log Kow>6; MEF high log Kow>8; MST high log Kow.8) in corresponding concentrations in the effluent ranging from 0.9 – 11 pg/L, and detected above the limit of detection (LOD, 0.2 pg TEQ/L) in 2 fine fractions (MCD medium Log Kow 5-6; MCD high Log Kow>8). Applications of the acidic cleanup resulted usually in a strong reduction of the DR-CALUX response to les than 2% of the original response (before acidic cleanup) for the MCD and MIJ fractions, 11-14% for the MEF fractions, and 46% for the MST fraction.

The concentrated refined fractions that tested above the LOQ (97 pg/TCDD-TEQ/liter sample) were analysed by GC-MSD. Using AMDIS to deconvolute the mass spectral data set, no undisputed identification of chlorinated compounds was achieved. Because of the complexity of the remaining data set of unidentified compounds, no further identification was done.

Additional verification with GC-NCI-MS, a technique more sensitive for chlorinated compounds, confirmed the predominance of non-chlorinated compounds and did not reveal significant amounts of chlorinated compounds above the limit of detection.

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Appendix I. Switching times for refined fractionation by reversed phase HPLC

To derive the (linear) relationship between log K_{ow} and retention time in a reversed phase HPLC system, a mixture of compounds covering a wide log K_{ow} range is used (see Table I.1). It includes a series of polyaromatic hydrocarbons (PAHs) and - for the purpose of the OVOC study - a number chlorinated compounds including PCBs.

Table I.1 Compounds used to derive the relationship between log K_{ow} and retention time.

Compound	log Kow	Key in fig. I.1
chloridazon	1.14	1
metalaxyl	1.65	1
benzothiazole	2.01	1
toluene	2.69	1
naphthalene	3.30	1
1,2-dichlorobenzene	3.43	1
1,3-dichlorobenzene	3.65	1
acenaphtene	3.92	1
acenaphtylene	3.93	1
1,2,4-trichlorobenzene	4.02	1
fluorene	4.18	1
phenanthrene	4.46	1
tetrachlorobenzene	4.60	1
fluoranthene	5.16	1
o,p'-DDD	5.87	2
PCB101	6.80	2
PCB138	7.44	2
octochlorostyrene	7.46	2
PCB180	8.27	2
benz(a)anthraceen	5.76	3
benzo(b)fluorantheen	5.78	3
chrysene	5.81	3
benzo(k)fluorantheen	6.10	3
benz(a)pyreen	6.13	3
benzo(ghi)peryleen	6.63	3
indeno(1,2,3-cd)pyreen	6.70	3
dibenzo(a,h)anthraceen	6.75	3

Using the HPLC method described in Paragraph 2.2.1, the relative retention time of each compound is determined and plotted against its log K_{ow}. At log K_{ow} values above log K_{ow} 5, two distinct patterns can be observed for the higher mass PAHs (4 condensed rings or more, shown in green) and the chlorinated compounds (shown in orange). The chlorinated compounds have considerably shorter relative retention times than the PAHs at the same log K_{ow}. As the OVOC study primarily focuses on chlorinated compounds, the linear relationship for the determination of the switching times of the fraction collector is derived using the retention times of the chlorinated compounds at higher log K_{ow} values, which is shown in Figure I.1. In Figure I.1, the data points of the different groups of compounds are coloured as indicated in Table I.1.

With the linear relationship, the switching times of the fraction collector for the collection of the different log K_{ow} intervals were derived, as shown in Table I.2.

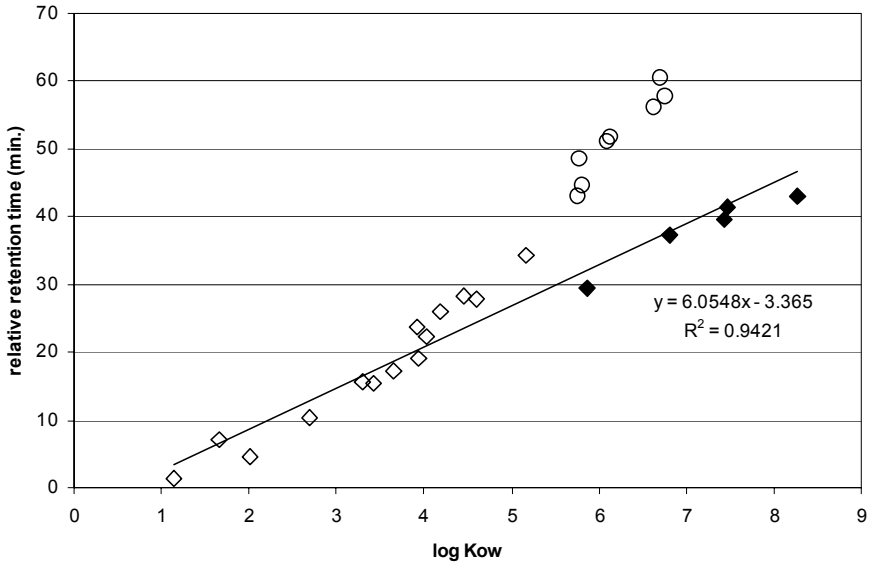

Figure I.1 Linear relationship between relative retention time and log K_{ow}.

Symbols:
(1) open diamonds: small PAHs (< 4 rings) and test-mixture, Log Kow < 5.
(2) open circles: chlorinated compounds with Log Kow > 5.
(3) black diamonds: large PAHs (4 or more rings).

Table I.2 Switching times for the fraction collector derived from the linear relationship between log K_{ow} and relative retention time.

Original fraction from screening study	Log K_{ow} interval for fine fractionation	Switching times for fraction collection (min)
Medium log K_{ow}	3-4	17.4-23.5
	4-5	23.5-29.5
	5-6	29.5-35.5
	>6	35.5-92.0
High log K_{ow}	5-6	29.5-35.5
	6-7	35.5-41.6
	7-8	41.6-48.0
	>8	48.0-92.0